Genomics 96 (2010) 67–72

Contents lists available at ScienceDirect

Genomics

journal homepage: www.elsevier.com/locate/ygeno

Presence of 5-methylcytosine in CpNpG trinucleotides in the human genome

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ARTICLE INFO

Article history:
Received 18 May 2009
Accepted 23 March 2010
Available online 9 April 2010

Keywords:
Methylation
CpNpG
DNA methyltransferase
DNMT1
DNMT3a

ABSTRACT

While the methylation machinery of mammalian cells has been shown to be capable of both maintenance and *de novo* methylation at CpNpG sites, CpNpG methylation in the human genome has not been demonstrated. Here, we report the first observation of 5-methylcytosines in CpNpG triplets in the human genome. We identify the existence of CpNpG methylation in a number of genes which contain trinucleotide repeat regions, including the androgen receptor (*AR*). We further analyzed DNA extracted from primary tissue samples and found the same pattern of CpNpG methylation. To confirm our results, we performed Southern blot analysis by analyzing the cleavage sites of restriction enzymes within exon 1 of the *AR* gene and found direct evidence of the presence of 5mCs in CpNpG triplets in the human genome. Our results also suggest that this methylation pattern may be due to the human DNA methyltransferases DNMT1 and DNMT3A. Although the functional significance needs to be tested further, the discovery of inheritable CpNpG methylation in the human genome may have important implications in our understanding of gene regulation and of the development of various diseases, including cancer.

Introduction

Methylation of cytosine residues in prokaryotic and eukaryotic genomes is an important epigenetic mechanism in gene regulation [1–4]. It is generally believed that cytosine methylation occurs exclusively in CpG dinucleotides in mammalian genomes [4–6]. In contrast, in plant genomes, 5-methylcytosines (5mC) in CpNpG triplets is as frequent as in CpG dinucleotides, where N can be any base and the methylcytosine occurs at a symmetrical sequence [7]. The existence of CpNpG methylation in mammalian genomes *in vivo* remains contentious. While data derived from nearest neighbor analysis suggests that a considerable proportion of 5mC was not located exclusively in CpG dinucleotides, the data from genomic sequencing of mammalian DNA has not uncovered abundant methylation at non-CpG sites [8–13]. These consistencies have been partially answered by Clark et al. who demonstrated that the methylation machinery of mammalian cells has been found to be capable of both maintenance and *de novo* methylation at CpNpG sites [14]. However, they failed to demonstrated CpNpG methylation in mammalian genomes although such methylation was identified within a stably integrated segment of plasmid DNA. In this report, using bisulfite sequencing and Southern blot analysis, we provide direct evidence demonstrating the presence of 5mCs in CpNpG triplets in the human genome.

Results

Presence of CpNpG methylation in cell lines

To determine if CpNpG methylation is present in the human genome, we screened 29 genes; 22 were encoded in the nuclear genome while 7 were encoded in the mitochondrial genome (Table 1). Fifteen of these genes contained a CpNpG trinucleotide repeat region. Using the bisulfite sequencing approach, we analyzed the methylation status of these genes in a number of cancer cells lines of different origins. The large array of cell lines was used to determine if CpNpG methylation was tissue-specific, a phenomenon that is seen with some CpG methylation. Genomic DNA from the 35 cancer cell lines were treated with sodium bisulfite which converted unmethylated cytosine residues to uracils while methylated cytosine residues resisted such treatment. Sodium bisulfite-modified DNA was amplified using PCR primers designed according to sequences not affected by methylation. Of these 29 genes, 6 demonstrated extensive CpNpG

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doi:10.1016/j.ygeno.2010.03.013

Table 1
Summary of genes examined, primer sequences for bisulfite sequencing, and CpNpG methylation status. MT: mitochondrial genome. M: CpNpG methylation present. U: no CpNpG methylation present.

Gene	Locus	Forward primer (5′→3′)	Reverse primer (5′→3′)	Methylation status
AIB1 (NCOA3)	20q12	AGGGTTTTTTTAATGTTTAAATGGT	ATCCTGCCAAAACCCATCCATACT	M
AR	X	AAGATTTATTGAGGAGTTTTTTAGAAT	AATAACCTATAAAACCTCTACAATAAA	M
ASH1 (ASCL1)	12q22–q23	GAAAGTTTTGTTAAGATGGAGAG	CAAAACCCAAATTAACCAAC	M
BHLHB5 (BHLHE22)	8q13	GAGGGGTGTTTTAATGTTTATT	CCTTTTACTCTTTAAATTTCTTACT	U
BRCA1	17q21	AGTTTATGGGAAGTAGTTATGTATTTTAGG	CTAAAATAATATTCCTAAAATACCTTTACC	U
DMPK	19q13.2–q13.3	TTGTTTTGAGTGTGGGTTTT	AACCCAACTTACTACCTTCC	U
DRPLA (ATN1)	12p13.31	GTGTGGAGTTAGGGTTTTT	AAACCATTAAAACCTACTTAACTATAA	U
E2F4	16q22.1	GGGGGTTATTATTGTAGTGA	CCTTAATAAACTCAAAAAAA	U
GAGE7B (GAGE12I)	X	GTGGGATTTTTTTATTTTTAAT	ACTACCTCACAACTCCCTAAC	U
H19	11p15.5	TGTTTTTTGAGGGGAGATAGTGGTTTGGGA	AATATCCCTCATAAACTC	U
HD (HTT)	4p16.3	GATTTTGGAAAAGTTGATGAAGGT	AATATCCCTCATAAACTC	U
NOTCH4	6p21.3	GGATTGGGGTTTGGAGAA	TAAAAATCTCCTCCATCCA	U
POLG	15q25	AAAAGAAGTTAAGTTGGAGTTT	ACATCTCCCCTCCTTACC	U
RAI1	17p11.2	TTATTTTTATAAGAGTTGTA	TAAAAAATTTCCTAAACATA	U
SCA1 (ATXN1)	6p23	AGTTTATTGGGTTTTTTTAA	ACCCCCAAATAATAAACCCC	M
SCA2 (ATXN2)	12q24	TGTATGGGTTTTTTATTATG	CCTCATATTTACATAAATTCCATCAA	U
SCA3 (ATXN3)	14q24.3–q31	TGATAGGTTATTTTGTGATGAAA	CTAAATCACTCCCAAATACTCC	M
SCA7 (ATXN7)	3p21.1–p12	GGTTGTGGATGATGTTAGGG	TAAAAACCTCAACCCACAAA	U
SCA8 (C10orf2)	13q21	AGTATGAGGAAGTATGGAAAAA	TCCCAATTCCTTAACTAAACC	M
SCA12 (PPP2R2B)	5q31–q33	AGGGGAGGAGGTTGGAAGAG	ACTCACCCTCACACCCACAC	U
SCA17 (TBP)	6q27	GTTTATTTTGGAAGAGTAATAAAGG	AATACCTAACCTAAAATTCCCTA	U
ATP6 (MT-ATP6)	MT	GGGTGTAGTGATTATAGGTT	AACTCTTCCAATTAAATACA	U
COX1 (MT-CO1)	MT	GGGAATTATTTTTATTTTGGAG	AAAAACCAATTAATATCATAACTCA	U
COX2 (MT-CO2)	MT	TTGGTTATTAATGGTATTGAATTT	AACATAAAACTATAATTTACTCCACA	U
COX3 (MT-CO3)	MT	GATTTTTGGTTTAGTTATGTGA	AAACCACATCTACAAAATACCA	U
CYTB (MT-CYB)	MT	TTATTTTGAGGGGTTATAGTAAT	CAAAAACTCCTCCTAATTTAT	U
ND1 (MT-ND1)	MT	AATTGTAATGGTATTTTTAATGTTT	TCTAATATTAAAACCTAAAACTAATTC	U
ND5 (MT-ND5)	MT	ATTTATGTGTTTAGATTAAGAAGTT	TTTTAAATAATCCTCCTATTTTTC	U

methylation (Table 1, Fig. 1a). As seen in the sequencing results for the androgen receptor (*AR*) gene, one of the two alleles from each cell line was unmethylated in most of the CpG sites and in all CpNpG sites in the genomic region (Figs. 1a and b). The other allele showed extensive CpG methylation consistent with the nature of an inactivated X chromosome. In addition, we observed several 5mCs in the CpNpG sites in the latter allele, including three consecutive CTG sites and two CAG sites (Fig. 1a). Interestingly, we observed this methylation phenomenon only in genes with trinucleotide repeat regions (Table 1, Figs. 1a and b). Furthermore, we found that this pattern of methylation was associated with the trinucleotide repeat region; sequencing of the other regions did not demonstrate 5mCs in CpNpG sites (data not shown). It should be noted that the sequencing did not demonstrate tissue specificity; while CpNpG methylation was not observed in every cell line examined, it was seen in cell lines of differing origins. For example, methylation of the trinucleotide region of the *AR* gene was observed in both lung cancer cell lines and prostate cancer cell lines (data not shown).

Presence of CpNpG methylation in tissues

Because it is believed that DNA methylation patterns can be significantly altered in cell culture conditions and in particular because DNA methylation density can be increased, we analyzed constitutional DNA extracted from 30 head and neck tumor samples, 30 lung tumor samples, and 8 normal lung tissue samples. Similar to our examination of CpNpG methylation in cancer cell lines, a large number of primary tissue samples were sequenced to determine if there was a difference in CpNpG methylation between normal and tumor samples. We observed CpNpG methylation in tissue samples as well (Table 1, Fig. 1b). However, the sequencing did not demonstrate a difference in CpNpG methylation between normal tissue samples and tumor samples. The result indicates that the 5mCs in CpNpG sites are found not only in culture conditions but are also found naturally in the human genome. These data, therefore, provide direct evidence of the presence of 5mCs in CpNpG triplets in the human genome.

Confirmation of CpNpG methylation by Southern blot analysis

To confirm our results, we performed Southern blot analysis. As shown with the *AR* in Fig. 2a, a roughly 3.8-kilobase (kb) fragment including the relevant methylation region could be generated by AccI (GT↓MKAC) digestion. Four PstI sites (CTGCA↓G) were identified within the 3.8-kb fragment (located at 34 bp, 856 bp, 1125 bp, and 1580 bp distant to the proximal AccI site) (Fig. 2a). Because the restriction enzyme PstI is methylation-sensitive (it cannot cleave the site if the 5′ cytosine is a 5mC), we predicted that the PstI site located at 856 bp distant to the proximal AccI site could not be cleaved due to CTG methylation. Genomic DNA samples were digested by AccI alone or AccI plus PstI, followed by Southern blot analysis using a 572-bp DNA probe located between the first two PstI sites (Fig. 2a). When DNA was digested by AccI alone, only a single 3.8-lb band could be detected in all four specimens as predicted (Fig. 2b). When DNA was digested by AccI and PstI, two bands, 0.8-kb and 1.1-kb, were observed in each of the specimens (Fig. 2b). This pattern was consistent with our prediction that the second PstI site could not be cleaved by PstI in one of the two alleles because of the presence of a 5′ 5mC (Fig. 1b). The lack of any band at the 3.8-kb and 1.5-kb positions indicated that the most distal PstI site was completely unmethylated in both alleles, and that enzymatic cleavage was complete.

CpNpG methylation may be mediated by DNMT1 and DNMT3a

There are four members of the human DNA methyltransferase (DNMT) family: DNMT1, DNMT2, DNTM3A, and DNMT3B. While the methylation machinery of mammalian cells has been shown to be capable of both maintenance and *de novo* methylation of CpNpG sites, it has not been conclusively determined which DNA methyltransferase is responsible [14]. To determine which DNMT is associated with

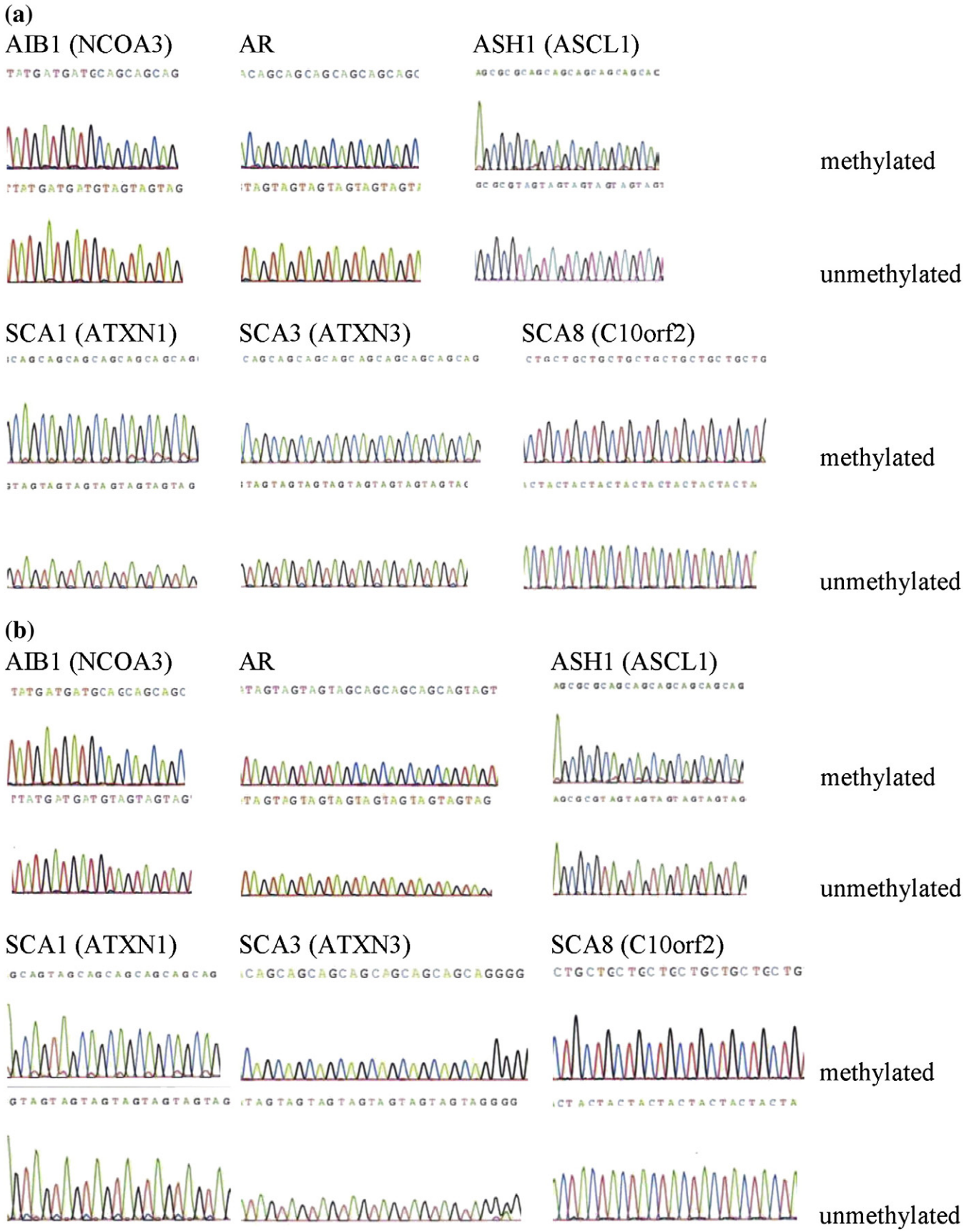

Fig. 1. Bisulfite sequencing analysis. (a) Representative bisulfite sequencing results in cell lines are shown for each gene which demonstrated CpNpG methylation. (b) Representative bisulfite sequencing results in primary tissue samples are shown for each gene which demonstrated CpNpG methylation.

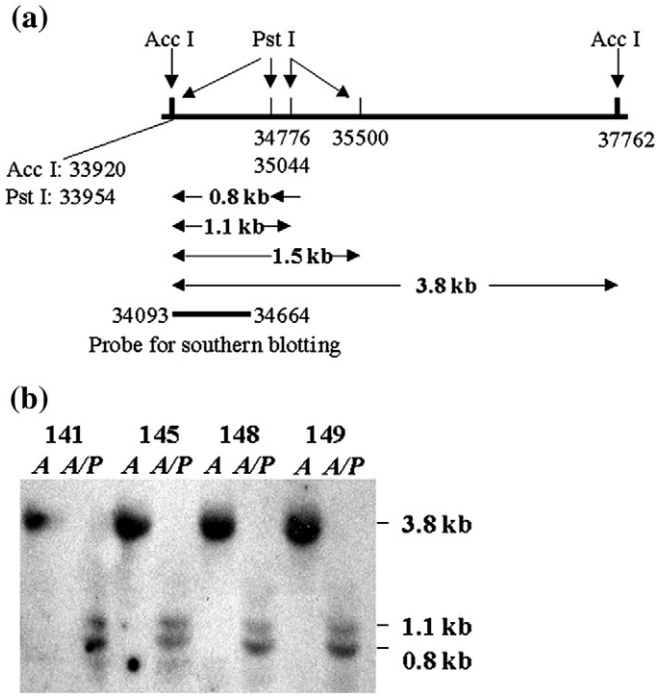

Fig. 2. Southern blot analysis for DNA methylation status within exon 1 of the *AR* gene. Constitutional DNA from 4 women were analyzed. (a) Restriction enzyme map in the region of the *AR* gene. Positions of each enzymatic cleavage site are indicated according to sequences in GenBank accession number AL049564. Approximate distances from the restriction enzyme cleavage sites to the first AccI site are indicated. The location of the probe for hybridization is also indicated. (b) The results of Southern blot analysis. A, genomic DNA cleaved only with AccI; A/P, genomic DNA cleaved with both AccI and PstI. The approximate sizes of DNA fragments are indicated on the right side.

CpNpG methylation in humans, siRNA were designed for DNMT1, DNMT2, DNMT3A, and DNMT3B. Three lung cancer cell lines, NCI-H1944, NCI-H1975, and NCI-H292, were treated with DNMT siRNA at a concentration of 75 nM for 10 days. RNA, DNA, and protein were extracted on days 1, 5, and 10. After 10 days of treatment, we observed substantial depletion in expression of all four DNMTs (Fig. 3a). We then analyzed the methylation status of the 6 genes exhibiting CpNpG methylation in the siRNA-treated cells using the bisulfite sequencing method. After 1 day of treatment, there was no change in the methylation status of these genes (Fig. 3b). After 10 days, however, we observed considerable demethylation of CpNpG sites in cells treated with DNMT1 and DNMT3A siRNA while no change in the methylation status was observed in cells treated with DNMT2 or DNMT3B siRNA (Fig. 3b). These results seem to implicate DNMT1 and DNMT3A in CpNpG methylation.

Discussion

While the methylation machinery of mammalian cells has been shown to be capable of both maintenance and *de novo* methylation at CpNpG sites [14], CpNpG methylation in mammalian genomes has not been demonstrated. While studying the methylation patterns in the human androgen receptor gene for clonality analysis, we had identified initial evidence of CpNpG methylation in tumor cell lines [17]. Through a further expansion of this initial observation, this report provides definitive evidence for the presence of 5mCs in CpNpG triplets in the human genome. Using bisulfite sequencing, abundant CpNpG methylation was found in cell lines and tissues. Our results also demonstrated that CpNpG methylation occurred predominantly in genes with polymorphic repeat regions and was seen only in the trinucleotide repeat regions. Furthermore, to avoid possible sequence and other artifacts,

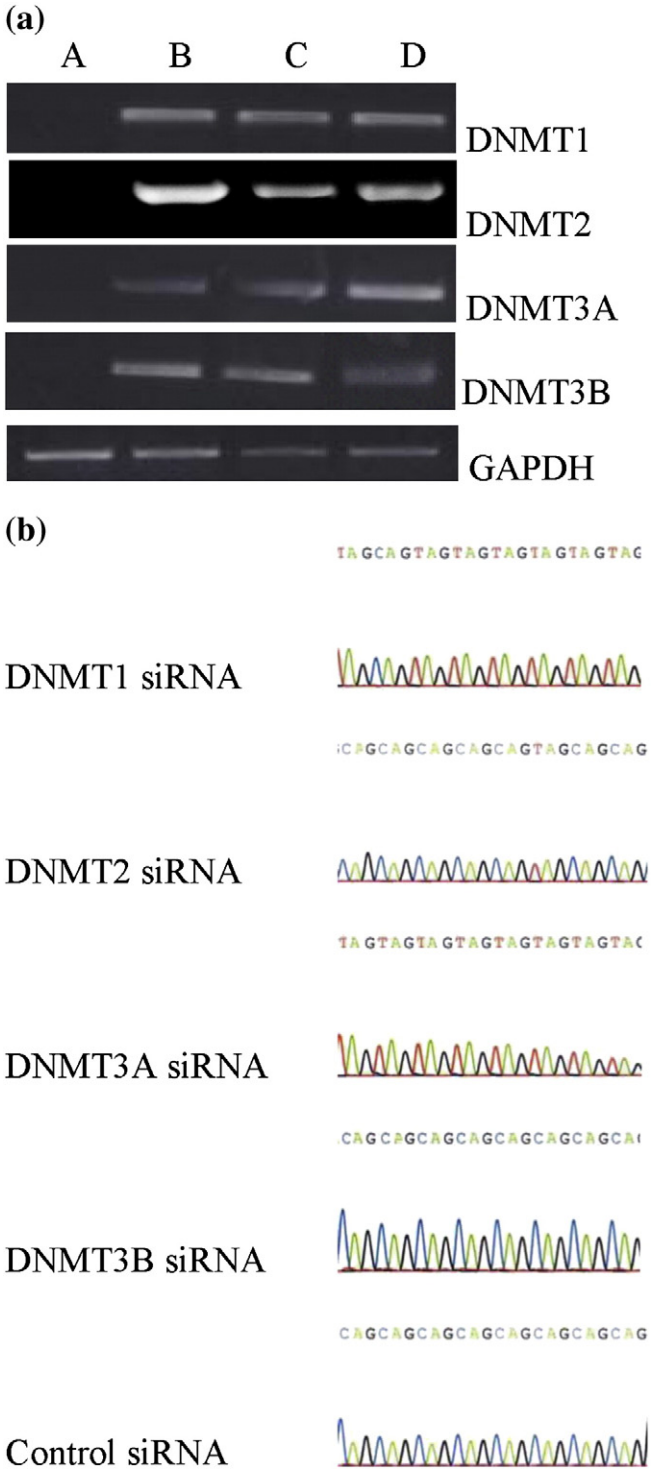

Fig. 3. DNMT-specific inhibition and demethylation. (a) H1944 cells were treated for 10 days with 75 nM of siRNA designed against DNMT1, DNMT2, DNMT3A, or DNMT3B (lane A) and with controls of scrambled siRNA (lane B), transfection reagent alone (lane C), and completely untreated (lane D). After 10 days, there was significant depletion of the respective DNMTs, both at the level of mRNA and protein. Treatment with the scrambled siRNA and the transfection agent alone did not deplete any of the DNMTs. GAPDH and β-actin were used as controls for loading. (b) The CpNpG methylation status of the *AR* was determined through sodium bisulfite sequencing of the DNA from these siRNA-treated cell lines. Cells treated with DNMT1 and DNMT3A siRNA demonstrated demethylation of exon 1 of the *AR* while cells treated with DNMT2 and DNMT3B siRNA and the controls remained methylated.

our results were confirmed by Southern blot analysis. Finally, treatment with various siRNA designed against the DNMT1, 3A, and 3B determined that CpNpG methylation appears to be mediated by

DNMT1 and DNMT3A. To our knowledge, this is the first report unequivocally demonstrating the evidence of CpNpG methylation in mammalian genomes, and is certainly consistent with a prior prediction from the finding of methylation machinery in the mammalian cell lines [14].

So far, it is generally believed that non-CpG dinucleotide methylation (cytosine methylation at CpA or CpT) is prevalent almost exclusively in embryonic stem cells and negligible tissues and that it is mediated by DNA methyltransferase 3A [16]. However, the functional significance of non-CpG dinucleotide methylation during the early stage of development is quite uncertain. Likewise, although recent studies showed that methylation of the outer cytosine residue of a CpCpG site can block Sp1 binding, resulting in transcriptional suppression [17], the functional significance of symmetrical CpNpG methylation has not been explained. Thus far, we have observed CpNpG methylation only in trinucleotide repeat regions and it is possible that methylation of the repeat region plays a protective role against the expansion of these trinucleotide repeats. While the significance of this pattern of methylation has yet to be determined, the discovery of inheritable CpNpG methylation in the human genome may have important implications in our understanding of gene regulation and of the development of diseases, including the carcinogenic process and inheritable neurological conditions.

Materials and methods

Cell lines and tissues

We analyzed a panel of cancer cell lines: 16 lung cancer cell lines (A549, H1299, H1437, H1563, H1568, H1650, H1703, H1838, H1944, H1975, H2030, H226, H292, H23, H522, and H838), 10 head and neck cancer cell lines (011, 012, 013, 019, 022, 028, Fadu, KYSE30, KYSE410, and KYSE520), and 4 colorectal cancer cell lines (DLD, HCT116 p53 +/+, HCT116 p53−/−, and SW480). The lung cancer cell lines and nine head and neck cancer cell lines (011, 012, 013, 019, 022, 028, KYSE30, KYSE410, and KYSE520) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin. The head and neck cancer cell line FaDu was cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS, penicillin, and streptomycin. The colorectal cancer cell lines HCT116 p53 +/+, HCT116 p53−/−, and DLD1 were cultured in McCoy's 5A medium supplemented with 10% FBS, penicillin, and streptomycin while SW480 was cultured in Leibovitz L-15 medium with 10% FBS, penicillin, and streptomycin. The lung cancer cell lines as well as FaDu, DLD1, and SW480 were purchased from the American Type Culture Collection. The colorectal cancer cell lines HCT116 p53 +/+ and HCT116 p53−/− were generously provided by Dr. Bert Vogelstein of The Johns Hopkins University while the head and neck cancer cell lines KYSE30, KYSE410, and KYSE520 were kindly provided by Dr. Shimada of Kyoto University. The head and neck cancer cell lines 011, 012, 013, 019, 022, and 028 were developed from the culture of primary HNSC in the Division of Head and Neck Cancer Research at The Johns Hopkins University.

30 primary lung tumor tissues, 30 primary head and neck tumor tissues, and 8 primary normal lung tissues were collected after surgical resections with prior consent from patients in The Johns Hopkins University Hospital as per institutional review board-approved protocols.

DNA extraction and bisulfite sequencing

Genomic DNA was extracted using phenol-chloroform extraction and ethanol precipitation in the presence of glycogen. One microgram of DNA from each cell line or tissue was used in the initial step of chemical modification. Briefly, DNA was denatured by NaOH and treated with hydroquinone and sodium bisulfite (Sigma, St. Louis, Missouri). After purification using Wizard DNA purification resin (Promega, Madison, Wisconsin), the DNA was treated again with NaOH. After precipitation, the DNA was recovered in water and ready for PCR using specific primers for amplifying both methylated and unmethylated sequences of each gene. The primer sequences are listed in Table 1. PCR reactions were carried out in a 50-μl volume containing about 50 ng of modified DNA, 3% dimethylsulfoxide, 200 μM of dNTP, 1.5 mM of MgCl2, 0.4 μM of PCR primers, and 1.25 U of *Taq* DNA polymerase (GIBCO BRL, Gaithersburg, Maryland). The DNA was amplified for 35 cycles at 95 °C for 30 s, 60 °C for 60 s, and 70 °C for 60 s followed by a 5-min extension at 70 °C in a temperature cycler (Hybaid, Omnigene, Woodbridge, New Jersey) in 500-μl plastic tubes. PCR products were separated on 2% agarose gels and visualized after staining with ethidium bromide. For each DNA sample, primer sets for methylated DNA and unmethylated DNA were analyzed.

A TA cloning kit (Invitrogen, Carlsbad, California) was used to clone the AR gene segment. One μl of fresh PCR products containing both methylated and unmethylated sequences were cloned into the TA vector as specified by the manufacturer. For sequencing, 500 ng of plasmid DNA containing inserts was used as a template for a reaction with an Ampicycle sequencing kit (Perkin Elmer, Boston, Massachusetts). The T7 primer for vector sequence was end labeled. Cycling conditions were followed as specified by the manufacturer.

Southern blot analysis

Genomic DNA was isolated from cell lines and from lung tissue samples from female patients by proteinase-K digestion (0.1 mg/ml) overnight at 50 °C followed by phenol-chloroform extraction and ethanol precipitation. For AccI digestion, 10 μg of genomic DNA from each sample was digested using restriction enzyme AccI (20 U) (Promega, Madison, Wisconsin) for 2 h at 37 °C in a 50-μl reaction volume and additional 10 U were added for an overnight digestion. For AccI plus PstI digestion, AccI digested genomic DNA was further digested with 10 U of PstI (GIBCO BRL, Gaithersburg, MD) for 2 h and additional 10 U of the enzyme was added for an overnight digestion. Digested genomic DNA from each sample was separated by electrophoresis on a 0.8% agarose gel. DNA was transferred to a nitrocellulose membrane (Amersham Life Science, Inc., Arlington Heights, Illinois) and then fixed by UV cross-linking. Following prehybridization in Rapid-Hyb buffer (Amersham Life Science, Inc.) for 1 h, the membrane was hybridized for 3 h with a radioisotope-labeled DNA probe. The Probe was synthesized by PCR amplification of a 572-bp DNA fragment from positions 34,093–34,664 (GenBank accession number: AL049564) (Fig. 2A) and labeled with γ[32p]dGTP using a random primer labeling kit (Promega) according to the manufacturer's protocol. The membrane was washed sequentially with 1× SSC/0.1% SDS at (65 °C for 30 min.), then 0.1× SSC/0.1% SDS (65 → °C for 30 min.) and exposed to autoradiography films.

siRNA treatment

siRNA were synthesized using the Silencer siRNA Construction Kit (Ambion) according to the manufacturer's instructions. The sequences of the DNMT1 siRNA templates are 5′-ACACATGTGAACG-GACAGATTCCTGTCTC-3′ (antisense) and 5′-TCTGTCCGTTCA-CATGTGTTTCCTGTCTC-3′ (sense). The sequences of the DNMT2 siRNA templates are 5′-AAGACGATTGAAGGCATTACACCTGTCTC-3′ (antisense) and 5′-AATGTAATGCCTTCAATCGTCCCTGTCTC-3′ (sense). The sequences of the DNMT3A siRNA templates are 5′-AAG-CACCCCCCGGTGGAAAGCCCTGTCTC-3′ (antisense) and 5′-AAGCTTTC-CACCGGGGGGTGCCCTGTCTC-3′ (sense). The sequences of the DNMT3B siRNA templates are 5′-CTCTAGGCATCCGT-CATCTTTCCTGTCTC-3′ (antisense) and 5′-AGATGACGGATGCCTA-GAGTTCCTGTCTC-3′ (sense). The sequences of the scrambled siRNA

templates are 5′-AATCGACCTCTGGAGCTAGCGCCTGTCTC-3′ (antisense) and 5′-AACGCTAGCTCCAGAGGTCGACCTGTCTC-3′ (sense). Cells were transfected with 75 nM of siRNA using Lipofectamine (Invitrogen) according to the manufacturer's instructions.

RNA extraction and RT-PCR analysis

Total RNA was extracted with Qiazol (Qiagen). Samples were treated with DNase I before reverse transcription using random priming and Superscript Reverse Transcriptase (Life Technologies), according to the manufacturer's guidelines. DNMT1, DNMT2, DNMT3A, and DNMT3b RT-PCR analyses were performed as previously described [15,16]. As an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified with 24 cycles to ensure cDNA quality and quantity for each RT-PCR. PCR was performed in a DNA thermal cycler (Perkins-Elmer Co.). The final PCR products were resolved on a 2% TBE agarose gel and visualized using ethidium bromide staining.

Western blot analysis

Cells were lysed in 100 µl of M-PER cell lysis buffer (Pierce) supplemented with 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium orthovanadate, and complete protease inhibitor cocktail (Roche Molecular Biochemicals) according to the manufacturer's instructions. Protein concentration was determined by the bicinchoninic acid assay (Pierce). Equivalent amounts of protein (50 µg/lane) were electrophoresed on 8% SDS–polyacrylamide gel. The gel was electroblotted onto nitrocellulose membrane and probed with the appropriate antibodies (DNMT1, 1:2000, New England Biolabs; DNMT2, 1:250, Abgent; DNMT3A, 1:500, Abcam; and DNMT3B, 1:500, Abcam). The membranes were visualized using chemiluminescence.

Acknowledgments

This work was supported in part by the National Institutes of Health/National Cancer Institute [to C. M., P50 CA96784-0]; the American Cancer Society [to L. M., RPG-98-054], a Cancer Center Grant [to M. D. Anderson Cancer Center, P30 CA 16620]; and the Tobacco Research Fund from the State of Texas [to M. D. Anderson Cancer Center].

References

[1] M.F. Lyon, X-chromosome inactivation: a repeat hypothesis, Cytogenet. Cell Genet. 80 (1998) 133–137.
[2] S.F. Wolf, D.J. Jolly, K.D. Lunnen, T. Friedmann, B.R. Migeon, Methylation of the hypoxanthine phosphoribosyltransferase locus on the human X chromosome: implications for X-chromosome inactivation, Proc. Natl. Acad. Sci. USA 81 (1984) 2806–2810.
[3] P.H. Yen, P. Patel, A.C. Chinault, T. Mohandas, L.J. Shapiro, Differential methylation of hypoxanthine phosphoribosyltransferase genes on active and inactive human X chromosomes, Proc. Natl. Acad. Sci. USA 81 (1984) 1759–1763.
[4] S. Tajima, I. Suetake, Regulation and function of DNA methylation in vertebrates, J. Biochem. (Tokyo) 123 (1998) 993–999.
[5] R.L. Sinsheimer, The action of pancreatic deoxyribonuclease. II. Isomeric dinucleotides, J. Biol. Chem. 215 (1955) 579–583.
[6] Y. Gruenbaum, R. Stein, H. Cedar, A. Razin, Methylation of CpG sequences in eukaryotic DNA, FEBS Lett. 124 (1981) 67–71.
[7] E.J. Richards, DNA methylation and plant development, Trends Genet. 13 (1997) 319–323.
[8] D.M. Woodcock, P.J. Crowther, W.P. Diver, The majority of methylated deoxycytidines in human DNA are not in the CpG dinucleotide, Biochem. Biophys. Res. Comm. 145 (1987) 888–894.
[9] R. Salomon, A.M. Kaye, Methylation of mouse DNA in vivo: di- and tripyrimidine sequences containing 5-methylcytosine, Biochim. Biophys. Acta 204 (1970) 340–351.
[10] R.H. Grafstrom, R. Yuan, D.L. Hamilton, The characteristics of DNA methylation in an in vitro DNA synthesizing system from mouse fibroblasts, Nucleic Acids Res. 13 (1985) 2827–2842.
[11] J. Nyce, L. Liu, P.A. Jones, Variable effects of DNA-synthesis inhibitors upon DNA methylation in mammalian cells, Nucleic Acids Res. 14 (1986) 4353–4367.
[12] M. Toth, U. Müller, W. Doerfler, Establishment of de novo DNA methylation patterns. Transcription factor binding and deoxycytidine methylation at CpG and non-CpG sequences in an integrated adenovirus promoter, J. Mol. Biol. 214 (1990) 673–683.
[13] S. Tommasi, J.M. LeBon, A.D. Riggs, J. Singer-Sam, Methylation analysis by genomic sequencing of 5′ region of mouse Pgk-1 gene and a cautionary note concerning the method, Somat. Cell Mol. Genet. 19 (1993) 529–541.
[14] S.J. Clark, J. Harrison, M. Frommer, CpNpG methylation in mammalian cells, Nat. Genet. 10 (1995) 20–27.
[15] M.Z. Fang, Y. Wang, N. Ai, Z. Hou, Y. Sun, H. Lu, W. Welsh, C.S. Yang, Tea polyphenol (−)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines, Cancer Res. 63 (2003) 7563–7570.
[16] S.-T. Pang, W.-H. Weng, A. Flores-Morales, B. Johansson, M.R. Pourian, P. Nilsson, A. Pousette, C. Larsson, G. Norstedt, Cytogenetic and expression profiles associated with transformation to androgen-resistant prostate cancer, Prostate 66 (2005) 157–172.
[17] S.J. Jang, L. Mao, Methylation patterns in human androgen receptor gene and clonality analysis, Cancer Res. 60 (2000) 864–866.